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Laboratory Experiment

Analysis of Protonation Equilibria of Amino Acids in Aqueous Solutions Using Microsoft Excel

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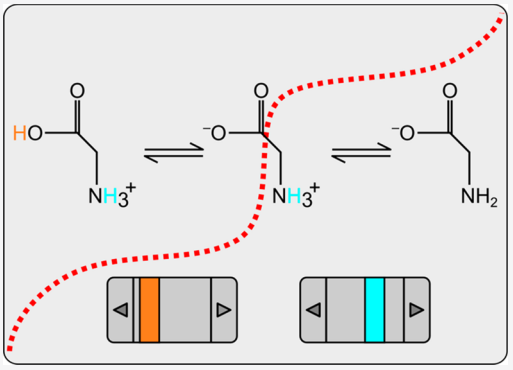
ABSTRACT: A Microsoft Excel workbook has been developed to simplify the quantitative analysis of experimentally measured titration curves for diprotic and triprotic amino acids such as glycine, arginine, histidine, and glutamate. Students perform the titration, enter the data into the worksheet, and manually adjust the resulting pK_a values to achieve the closest agreement between the experimental and calculated titration curves. At the same time, the students can observe changes in the speciation diagram that illustrates the transitions of differently protonated molecular species during the titration. Through a combination of graphical visualization and analysis, supported by computer software, students learn the most important aspects of the protonation equilibria of amino acid solutions. The knowledge gained can serve as the basis for understanding the behavior of more complex polyprotic acids in aqueous solution and the pK_a values of amino acid residues in proteins.



KEYWORDS: *Upper-Division Undergraduate, Graduate Education/Research, Biochemistry, Computer-Based Learning, Amino Acids, Equilibrium, pH, Titration/Volumetric Analysis*

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INTRODUCTION

Proton transfer in aqueous systems plays a fundamental role in many biochemical processes, such as enzyme catalysis,[1−3] conformational changes of macromolecules,[4−6] maintenance of pH gradients in cell compartments,[7] formation of some noncanonical DNA structures,[8] and others. The determination of pK_a values of biological molecules can be accomplished experimentally using FTIR, NMR, or Raman spectroscopic methods.[9−15] In some cases, pK_a values can be obtained using various mathematical models.[16−22] All these methods are too complicated to be performed by students in undergraduate and graduate degree programs. A conceptually and experimentally more accessible approach is to perform titrations of single amino acids[23,24] to determine the pK_a values of their functional groups. Simple titration curves are often recorded during a study in the majority of chemical and biochemical programs but titrations of amino acids, which are considered weak acids, are rather scarce. In contrast to monoprotic acids, it is not a trivial task to determine pK_a values of polyprotic amino acids quantitatively. For this reason, students are commonly encouraged to analyze titration curves semiquantitatively using a graphic method where they become familiar with basic features of titration curves such as buffering regions and isoelectric points. Naturally, a second, more advanced step is an exact quantitative analysis of the whole titration curve which would strengthen students' understanding of protonation equilibria. Such a quantitative analysis relates a mathematical description and the use of computer software to the experimental data, and therefore, it may significantly improve the understanding of these topics. Graphical simulations of acid−base pH titrations, using a computer spreadsheet, and applications for educational purposes are available.[25−30] A theoretical description of titration curves, using a direct single closed-form expression without assumptions and segmentations (dissection of titration curve into two or more segments), is usually problematic since it involves solving a high-order polynomial.[27,31] Less complex approaches for data fitting have been proposed that are based on indirect methods using simplified assumptions or functions without direct physical significance.[32−35] However, to our knowledge, there are presently no simple tools for students that would combine graphical simulation, theoretical description, and data fitting for amino acid titrations.

Here we present a laboratory experiment that involves titration of amino acids followed by a quantitative analysis of the titration curves using the Excel VBA macros implemented in the workbook developed for this purpose. Briefly, students first perform a pH-titration of glycine and one triprotic amino acid, for example, glutamic acid, histidine, or arginine. The

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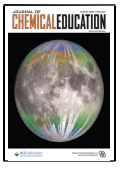

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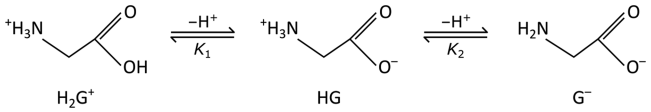
experimental data is then entered into the spreadsheet where students can determine the pK_a values by performing a "visual fit." In order that students link the changes of protonation state at the molecular level with the solution pH, the workbook generates the amino acid speciation diagrams that show how different states of protonation change throughout the titration. The laboratory assignment and student expectations are listed in the Supporting Information (SI), which also provides a possible strategy for further discussion of the results in the context of protein protonation equilibria. We include the described experiment as a part of the practical laboratory course for students in the first year of the biochemistry master's program.

THEORETICAL BACKGROUND

Diprotic Amino Acid

Glycine is the simplest amino acid and behaves as a weak diprotic acid in aqueous solutions. When the difference between the pK_a values is large enough (more than 2 pK_a units), a stepwise dissociation of the protons from the amino acid during titration with base can be assumed.[23] A stepwise model cannot be applied to all amino acids; for example, in the case of cysteine and tyrosine, the pK_a values are too similar.[23,36,37] The simplified protonation equilibrium between glycine species can be presented as shown in Scheme 1. In this scheme, K_1 and K_2 represent the equilibrium constants for each dissociation step and H_2G^+, HG, and G^- stand for species participating in this reaction. When the amino acid is prepared in a solution of a strong acid, for example, HCl, the starting state is a fully protonated one, that is, H_2G^+. In this case, during the subsequent additions of titrant, for example, NaOH, the whole titration curve of glycine is obtained as is shown below. Each dissociation step or constant is associated with the following equilibrium expressions

Scheme 1. Stepwise Model for a Diprotic Amino Acid[a]



[a]Equilibrium constants K_1 and K_2 denote the equilibria between differently protonated species, as shown by their molecular structures.

$$K_1 = \frac{[HG][H^+]}{[H_2G^+]} \Rightarrow [HG] = [H_2G^+]\frac{K_1}{[H^+]}$$

$$K_2 = \frac{[G^-][H^+]}{[HG]} \Rightarrow [G^-] = [HG]\frac{K_2}{[H^+]} = [H_2G^+]\frac{K_1K_2}{[H^+]^2} \quad (1)$$

The most important equations from the derivation of the final explicit expressions used for the calculation of amino acid titration curves are now presented. The complete derivation, which is carried out similarly to that in de Levie's paper,[31] is presented in the SI. The total concentration of glycine in a solution is constant and can be expressed as

$$c_T = [H_2G^+] + [HG] + [G^-] \quad (2)$$

If we substitute the last two concentrations by the expressions from eq 1, we obtain

$$c_T = [H_2G^+] + [H_2G^+]\frac{K_1}{[H^+]} + [H_2G^+]\frac{K_1K_2}{[H^+]^2} = [H_2G^+]\left(1 + \frac{K_1}{[H^+]} + \frac{K_1K_2}{[H^+]^2}\right) \quad (3)$$

We now define the molar fraction of H_2G^+ as

$$\alpha_{H_2G^+} = \frac{[H_2G^+]}{c_T} = \frac{[H^+]^2}{[H^+]^2 + K_1[H^+] + K_1K_2} \quad (4)$$

Similarly, we can derive molar fractions for the other two species

$$\alpha_{HG} = \frac{[HG]}{c_T} = \frac{K_1[H^+]}{[H^+]^2 + K_1[H^+] + K_1K_2} \quad (5)$$

$$\alpha_{G^-} = \frac{[G^-]}{c_T} = \frac{K_1K_2}{[H^+]^2 + K_1[H^+] + K_1K_2} \quad (6)$$

The expression for electrical neutrality of a solution during the titration is

$$[Na^+] + [H^+] + [H_2G^+] = [OH^-] + [G^-] + [Cl^-] \quad (7)$$

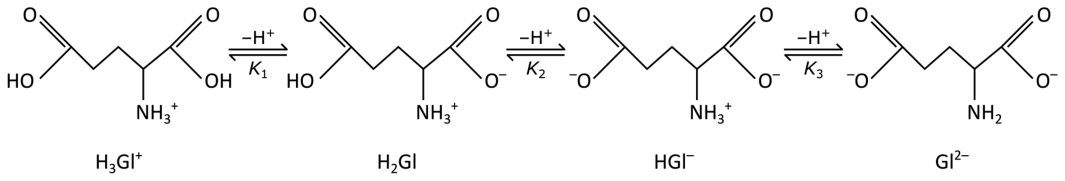
which can also be written as

$$c_{NaOH} + [H^+] + \alpha_{H_2G^+}c_T = \frac{K_w}{[H^+]} + \alpha_{G^-}c_T + c_{HCl} \quad (8)$$

where c_{NaOH} is the concentration of sodium ions from the titrant (NaOH), K_w is the ionic product of water, and c_{HCl} is the concentration of chloride ions in solution. By substituting the expressions for fractions in eq 6 by eq 4 and some manipulation of the equation, we obtain the final expression which is a polynomial of the fourth degree

$$[H^+]^4 + (c_{NaOH} + K_1 + c_T - c_{HCl})[H^+]^3 + (c_{NaOH}K_1 + K_1K_2 - K_w - c_{HCl}K_1)[H^+]^2 + (K_1K_2(c_{NaOH} - c_T - c_{HCl}) - K_1K_w)[H^+] - K_1K_2K_w = 0 \quad (9)$$

Scheme 2. Stepwise Model for the Triprotic Amino Acid[a]



[a]Equilibrium constants K_1, K_2, and K_3 denote the equilibria between differently protonated species, as shown by their molecular structures.

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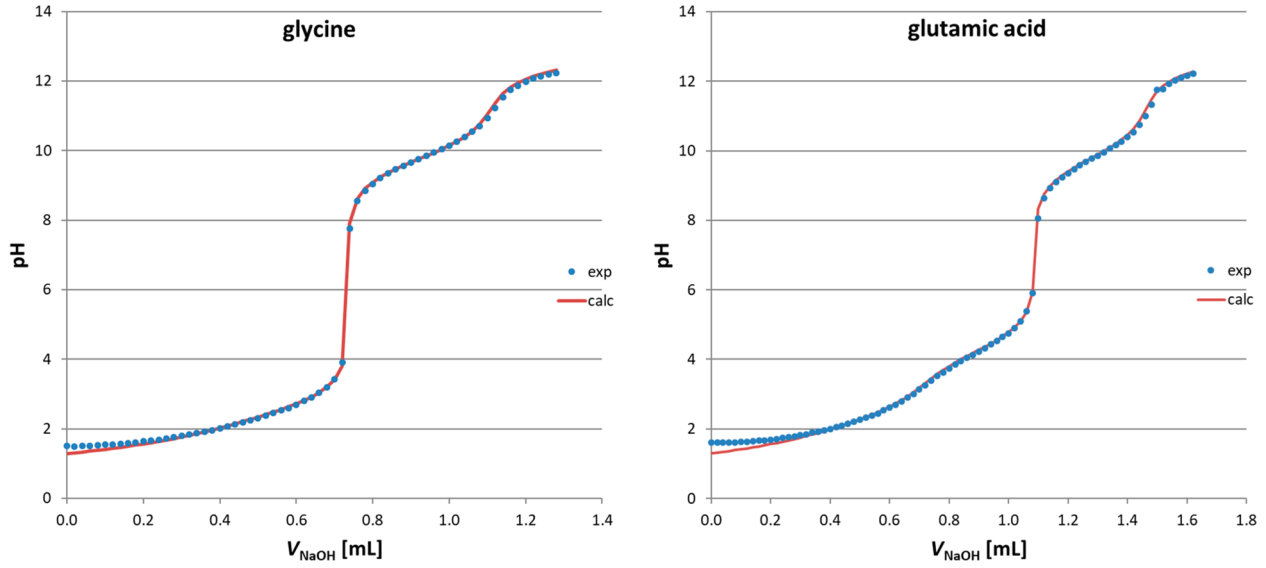

Figure 1. Titration curves for glycine and glutamic acid: Experimental (blue circles); calculated (red curve).

where $[H^+]$, K_1, and K_2 are the unknowns. Next, the roots of the polynomial (values of $[H^+]$ between 0 and 1) must be determined for every addition of titrant and given the assumed values of K_1 and K_2. This can be achieved by various methods. In our case, we use bisection, which was implemented in the VBA code of this Excel workbook (see the SI).

Triprotic Amino Acid

Here we discuss glutamic acid as an example of an amino acid with three titratable groups. In aqueous solution, glutamic acid behaves as a weak triprotic acid. The equilibrium between species is shown in Scheme 2, where K_1, K_2, and K_3 are the equilibrium constants for each dissociation step. By analogy to glycine, we can derive the final polynomial, which is now of the fifth degree and can be used for amino acids bearing positively charged side-chain groups

$$[H^+]^5 + (c_{NaOH} + K_1 + c_T - c_{HCl})[H^+]^4 + (c_{NaOH}K_1 + K_1K_2 - K_w - c_{HCl}K_1)[H^+]^3 + (K_1K_2(c_{NaOH} + K_3 - c_T - c_{HCl}) - K_1K_w)[H^+]^2 + (K_1K_2K_3(c_{NaOH} - 2c_T - c_{HCl}) - K_1K_2K_w)[H^+] - K_1K_2K_3K_w = 0 \quad (10)$$

where K_3 is an additional unknown. In the case of a negatively charged side-chain group the polynomial has a slightly different form, as presented in the SI.

EXPERIMENTAL SECTION

Experimental Setup and Titrations

Figure S1 shows the experimental setup for the laboratory experiment. A 25 mL beaker is placed on a magnetic stirrer fixed with a clamp and support stand. Using a glass pipet, the beaker is then filled with 15 mL of solution consisting of 0.05 M amino acid (glycine, glutamic acid, histidine, or arginine) in 0.1 M HCl. The beaker, which also contains a magnetic stirring bar, is then closed with a rubber stopper with two holes. The first one is used for a 2 mL micrometer dispenser (microburet) filled with a solution of 2 M NaOH; in the second, we place a combined pH glass electrode connected to a pH meter. Usually, the students have little experience using a microburet; therefore, they can start by titrating 0.05 M solution of acetic acid with 2 M NaOH before advancing to the titrations of amino acids. In this way, the students also determine the pK_a value of acetic acid, which also serves as a starting point for discussion (details in the SI). The titration is performed by adding 0.02 mL aliquots of titrant NaOH and measuring pH after each addition (it takes a few seconds for the value to equilibrate). The volume of the titrant and the corresponding pH values are entered into the Excel workbook for further analysis.

Data Analysis

The main Excel worksheet is divided into two sides: the left side is used for the analysis of glycine titration, and the right side for the triprotic amino acid, which is specified from the drop-down list (Figure S2). The cells highlighted in green are designated for the experimental data: volume of the titrant (V_{NaOH}) and the corresponding pH value (pH exp). Additional green cells are used to specify the solution conditions: the concentration of NaOH (c^o NaOH), the concentration of amino acid (c^o gly, for example), the initial volume of the solution (V sol.), and the concentration of HCl (c^o HCl).

In addition to the green input cells, five columns show calculated values. The first three show calculated values for concentrations of base (c NaOH), amino acid (c gly for example), and acid (c HCl). The last two columns show the calculated molar ratio between the base and amino acid (n_{NaOH}/n) and the theoretical pH values. After the students have entered the data, the scroll bars are unlocked, allowing them to adjust the pK_a values by moving the scroll bars. Given the assumed pK_a values, the implemented VBA code calculates the theoretical pH values and generates a theoretical titration curve that is shown together with the experimental one. By moving the scroll bars, students need to find pK_a values that result in the best agreement between calculated and experimental titration curve (Figure 1). In this way, they can observe how changes in pK_a values are reflected in the titration curve. Alternatively, they can also try the implemented automatic fitting by clicking the buttons located in the FIT frame on Sheet 1 (Figure S2).

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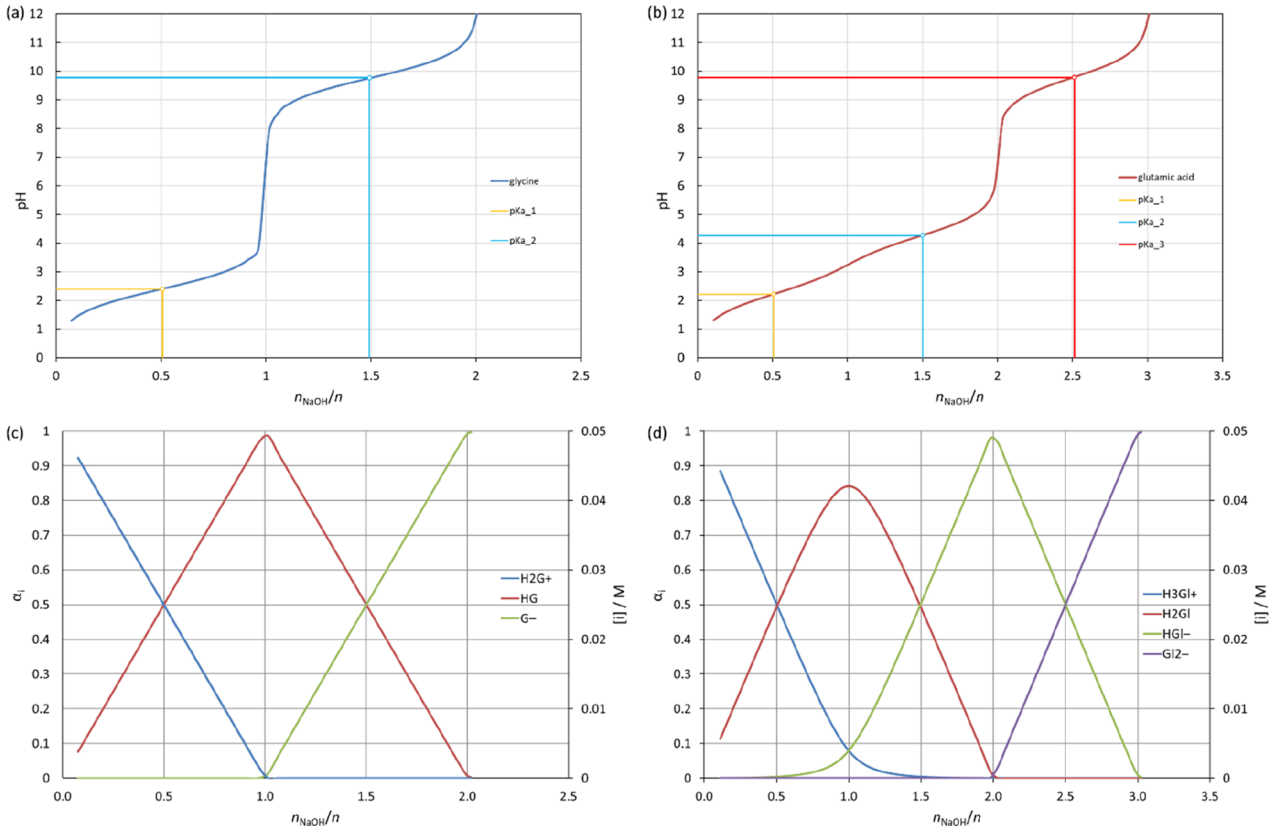

Figure 2. (a, b) Calculated titration curves for glycine and glutamic acid. The lines show the molar ratios at which the pK_a values can be determined graphically. (c, d) Species diagrams for glycine and glutamic acid show how molar fractions (α_i) and species concentrations ([i]) change as a function of n_{NaOH}/n.

Table 1. Comparison of pK_a Values Obtained for Different Amino Acids

	pK_a Values from Titration and Analysis Compared to Literature Values, by Amino Acid							
	Glycine		Glutamic Acid		Histidine		Arginine	
pK_a No.	Measured	Literature[a]	Measured	Literature[a]	Measured	Literature[a]	Measured	Literature[a]
1	2.39	2.4	2.21	2.2	1.99	1.8	2.11	1.8
2	9.77	9.8	4.26[c]	4.3	6.36[c]	6.0	9.35	9.0
3	–[b]	–[b]	9.78	9.7	9.52	9.2	13.56[c]	12.5

[a]See ref 39. [b]Glycine has only two pK_a values. [c]Values of side-chain groups.

After the students have obtained a satisfactory agreement between calculated and experimental titration curves and found the correct pK_a values (Figure 1), the program generates two additional charts (Figure 2). The first shows calculated pH values at given molar ratio n_{NaOH}/n (Figure 2a and b), and the second chart is a speciation diagram that shows how amino acid protonation states change as a function of n_{NaOH}/n (Figure 2c and d). The first chart appears under the sheet "Chart" and can be copied using the "Copy graph" button, while the second can be found in sheets "Species1" and "Species2". They can be used to complete the laboratory assignment (see the SI). The workbook is protected in such a way that students cannot change any values outside the green cells, the structure of the program, or the VBA code. The Excel workbook is also protected from saving (whenever the workbook is opened it appears as original) in order to avoid data exchange between students.

HAZARDS

Solutions of amino acids and 2 M NaOH cause irritation to skin and eyes, so titrations should be performed while wearing a safety coat, eye protection, and gloves.

RESULTS AND DISCUSSION

The experiment was conducted by a total of 98 students attending the first year of master's degree programs in biotechnology, chemistry, and biochemistry in the winter semester of 2019. All students completed the experiment, analyzed their data using the Excel workbook, and obtained reliable results. After the discussion of results and extended discussion about the pK_a values and their dependence on microenvironment (see the SI), students generally completed the exercise and finished laboratory assignments within 3 h and were satisfied by the knowledge about the topic gained.

In Table 1, we list the pK_a values obtained from the titrations of amino acids analyzed using the presented Excel workbook. The pK_a values of side-chain groups for glutamic

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acid, histidine, and arginine are 4.26, 6.36, and 13.56, respectively. Overall, the pK_a values determined for terminal and side chains agree with those reported previously (Table 1). We note that the obtained pK_a value for side chains of arginine is higher than is often cited in the literature (around 12); however, our value is in good agreement with that from Fitch et al., who recently revised this constant to 13.8 using the complementary approach of potentiometry and NMR spectroscopy.[38]

We suggest that the students start the laboratory experiment with glycine, which has only two titratable groups. Then they continue the experiment with a triprotic amino acid (e.g., glutamic acid, arginine, or histidine). In order to determine the pK_a value of the most acidic proton (pK_{a1}), the titration should start from the fully protonated state (H_2G^+ or H_3Gl^+, see Schemes 1 and 2), which can be achieved by dissolving amino acids in a solution of strong acid. Moreover, many amino acids are poorly soluble in water but their solubility increases with the concentration of the strong acid. For these reasons, we suggest that solutions are prepared in 0.1 M HCl. It is noteworthy that in such a setup the added base will be used for titration of the amino acid and the HCl. Consequently, the contribution of the base that has been consumed for the titration of HCl needs to be subtracted from the total volume of the base (V_{NaOH}). In other words, we have to obtain the number of moles of a base needed for the titration of an amino acid alone (n_{NaOH}) for further analysis. This is calculated automatically in the workbook when the students enter the starting solution conditions.

After the students have completed the titrations, we suggest that they inspect the titration curve to become familiar with its features and visually estimate the pK_a values.[24] Using these initial estimates, the students can use the scroll bars to obtain a reasonable agreement of the calculated curve with the experimental data and, thus, determine the pK_a values of all functional groups (including pK_{a1}). Alternatively, the best agreement can be achieved using the automatic fitting option. It is important that students can observe how different pK_a values affect the shape of the titration curve when adjusting the scroll bars (Figure 1). They can also simulate titration curves for other solution conditions (different concentrations of base, the volume of the solution, and the concentration of amino acid or added strong acid) and learn how the titration curve responds to such changes. Attention can be brought to the data in the acid region (pH < 2) where the experimental and theoretical curve systematically diverge (Figure 1). This is due to the acidic error of the glass electrode. Thus, from this part, students learn basic features of the titration curve, learn how to determine pK_a values, and test how different parameters affect the shape of titration curve.

After correct pK_a values are obtained, students can focus on the plots in Figure 2. These show the calculated titration curves as a function of n_{NaOH}/n for glycine and glutamic acid (Figure 2a, b). This representation, rather than having V_{NaoH} on the x-axis (Figure 1), helps students understand the course of titration better. For example, lines at given molar ratios illustrate how pK_a values can be determined graphically. Similar titration curves obtained for histidine and arginine are shown in the Figures S3 and S4. Figure 2c and d shows a species distribution (molar fractions (α) and concentrations of species ([i]) as a function of molar ratio (n_{NaOH}/n). These charts help students link the molecular structure with the observed proton equilibria. By using dissociation schemes presented in the Theoretical Background above, this can also be used to improve students' understanding of the buffering properties of amino acids. Thus, from this part, students learn how to interpret qualitatively all regions of the titration curve and to visually identify the pK_a values, buffer regions, and the isoelectric point (details in the SI).

The pK_a values determined can serve for further discussion on the impact of microenvironment on the pK_a values of ionizable side chains in proteins. As a starting point, the students can be asked why the pK_a value of glutamate carboxylic group differs significantly (difference in pK_{a1} = 2.21 and pK_{a2} = 4.26 is due to the proximal amino group). We then discuss an example of the active site of xynalase from *Bacillus circulans* in which the pK_a of glutamate (Glu 172) is 6.8,[9] which is higher than the value obtained from our titration (4.26). This is attributed to the Coulombic interactions between Glu 172 and the carboxylic group of Glu 78 in the active site (see Figure S5). Similar pK_a shifts of the residues can be found in numerous active sites of proteins, which can be attributed to attractive or repulsive Coulombic interactions between the internal ionizable residues. Other examples are presented in the SI. The lesson here is that students understand qualitatively how different interactions and microenvironments can upshift or downshift side-chain pK_a values in proteins.

CONCLUSIONS

Since the transfer of protons presents a part of complicated chemical and biochemical processes in aqueous solutions, laboratory titration experiments with single amino acids are the best starting point for introducing this subject to students in a biochemistry course. In this paper, we present an Excel workbook with implemented VBA code that enables the fitting of a calculated curve to the experimental titration data of diprotic (e.g., glycine) and triprotic amino acids (e.g., glutamic acid). The best agreement is accomplished by moving simple scroll bars where every change in fitting parameters (pK_a values) is instantaneously reflected on the corresponding graph. From the pK_a values obtained for glycine, students learn to assign correct pK_a values to both terminal and residual titrable groups of triprotic amino acids. Using the examples presented in this work, students will also be able to discuss the pK_a values of residues in proteins and their dependence upon the microenvironment.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available at https://pubs.acs.org/doi/10.1021/acs.jchemed.0c01144.

Excel workbook and experimental data for amino acids from Table 1 (ZIP)

Complete derivation of equations; additional figures; laboratory assignment; student expectations (PDF, DOCX)

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Notes

The authors declare no competing financial interest.

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